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Characterization and Functional Reconstitution of the Multidrug Transporter

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P-Glycoprotein, the multidrug transporter, is isolated from the plasma membrane of CH^RC5 cells using a selective two-step detergent extraction procedure. The partially purified protein displays a high level of ATPase activity, which has a high K_M for ATP, is stimulated by drugs, and can be distinguished from that of other membrane ATPases by its unique inhibition profile. Delipidation completely inactivates ATPase activity, which is restored by the addition of fluid lipid mixtures. P-Glycoprotein was reconstituted into lipid bilayers with retention of both drug transport and ATPase activity. Proteoliposomes containing P-glycoprotein display osmotically sensitive ATP-dependent accumulation of ^{3}H-colchicine in the vesicle lumen. Drug transport is active, generating a stable 5.6-fold concentration gradient, and can be blocked by compounds in the multidrug resistance spectrum. Reconstituted P-glycoprotein also exhibits a high level of ATPase activity which is further stimulated by various drugs. P-Glycoprotein therefore functions as an active drug transporter with constitutive ATPase activity.

KEY WORDS: Multidrug resistance; multidrug transporter; P-glycoprotein; detergent extraction; ATPase; inhibitors, active transport; colchicine; reconstitution; proteoliposomes

INTRODUCTION

Resistance to structurally unrelated multiple drugs is a leading cause in the failure of chemotherapy treatment for a large number of human cancers, including colon, kidney, and breast carcinomas, leukemias, and multiple myeloma. The overexpression of P-gp,[2] a 170-kDa plasma membrane protein, is responsible for a major type of multidrug resistance displayed by cell lines selected for growth *in vitro* in high levels of cytotoxic drugs (Gottesman and Pastan, 1993). Evidence is accumulating that P-gp expression in many tumors *in vivo* is associated with both poor response to chemotherapy and poor overall prognosis (Chin *et al.*, 1993).

Sequence analysis has shown that P-gp comprises two homologous halves, each consisting of six putative hydrophobic transmembrane segments and a consensus sequence for a nucleotide binding fold. These properties are shared by a large family of membrane transporters, known as the ABC, or traffic ATPase, superfamily (Higgins, 1992; Doige and Ames, 1993). Other members of the ABC superfamily include the cystic fibrosis transmembrane conductance regulator (CFTR), the yeast STE-6 a-factor peptide exporter, and the TAP-1/2 peptide transporters in the endoplasmic reticulum. P-gp exists as a small multigene family, with three members in rodents, and two in human. The class I and II P-gp isoforms have been shown to encode drug exporters, while the class III isoform is responsible for export of phospholipid into the bile

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[2] Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; *p*-CMBS, *p*-chloromercuribenzenesulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; EDTA, ethylene glycol bis(3-aminoethylether)*N,N,N',N'*-tetraacetic acid; MDR, multidrug-resistant; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; P-gp, P-glycoprotein; PI, phosphatidylinositol; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

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(Smit *et al.*, 1993), and appears to be a PC flippase (Ruetz and Gros, 1994).

Based on the observation that MDR cells export drugs in an energy-dependent fashion, and the ability of P-gp to be labelled with photoaffinity drug and ATP analogues, the protein is proposed to function as an ATP-driven drug efflux pump. However, biochemical evidence in support of this proposal has been obtained only in the last few years. One clinically important aspect of P-gp biochemistry is the existence of chemosensitizers, which are compounds able to reverse MDR by blocking the drug transport activity of P-gp (Ford and Hait, 1993). Commonly used chemosensitizers include drug analogs, verapamil (a Ca^{2+}-channel blocker), trifluoperazine (a calmodulin inhibitor), and cyclosporine A (an immunosuppressant). Several of these compounds have shown promise in clinical trials in combination with chemotherapeutic drugs.

The work carried out in our laboratory over the last few years has focussed on the biochemical characterization of P-gp with respect to both drug transport and ATPase activity. Our long-term objectives are to understand how the multidrug transporter interacts with and transports drug substrates, and how ATP hydrolysis is coupled to the transport process.

ISOLATION OF ATPase-ACTIVE P-GLYCOPROTEIN

The presence of one or two consensus sequences for a nucleotide-binding motif is characteristic of all proteins in the ABC superfamily. Tsuruo and co-workers were the first to report that affinity-purified P-gp showed low, but significant levels of ATPase activity (Hamada and Tsuruo, 1988a,b). Soon afterwards, our laboratory investigated various strategies for detergent solubilization and purification of P-gp from the plasma membrane of CH^RC5 cells (Doige and Sharom, 1991). This CHO cell line, which is a very useful *in vitro* MDR cell model, was previously selected for resistance to the drug colchicine (Kartner *et al.*, 1985). It expresses predominantly (>95%) the class I isoform of P-gp (Urbatsch *et al.*, 1994). During the course of our study, which used the zwitterionic detergent CHAPS, we noted that P-gp was especially difficult to extract from the plasma membrane. Even at a detergent concentration of 100 mM, only about half of the P-gp is removed from the membrane, whereas many other membrane proteins are completely solubilized. We exploited this observation to develop a selective, two-step extraction procedure for isolation of ATPase-active P-gp (Fig. 1A; Doige *et al.*, 1992).

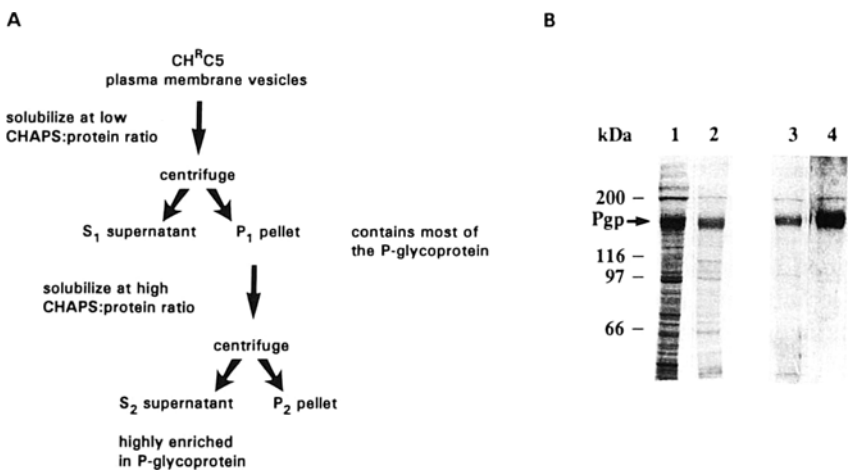

Fig. 1. Isolation and reconstitution of P-gp into proteoliposomes. (A) Schematic diagram showing the basic steps in the selective CHAPS extraction of P-gp (the S_2 fraction) from CH^RC5 plasma membrane. (B) SDS-PAGE analysis of partially purified P-glycoprotein (S_2 fraction) before and after reconstitution into proteoliposomes (reprinted with permission). Lane 1, CH^RC5 plasma membrane, 7.5 μg protein; Lane 2, partially purified S_2 fraction, 7.5 μg protein; Lane 3, reconstituted P-glycoprotein proteoliposomes, 0.75 μg protein; Lane 4, reconstituted P-glycoprotein proteoliposomes, 100 ng protein. Lanes 1–3 were from gels stained with Coomassie blue, whereas Lane 4 was from a gel stained with silver. The position of molecular mass markers is indicated on the left, and the band corresponding to P-glycoprotein (Pgp) is marked with an arrow. For full experimental details, see Doige *et al.* (1992) and Sharom *et al.* (1993).

CH^RC5 plasma membrane is treated with 25 mM CHAPS at a low detergent : protein ratio (1.5 : 1 w/w). After ultracentrifugation, the supernatant, which contains many other membrane proteins but only traces of P-gp, is discarded. The P-gp-enriched detergent-insoluble pellet is then extracted with 8 mM CHAPS at a high detergent : protein ratio (10 : 1 w/w). The supernatant (S_2) obtained after ultracentrifugation contains about 30–40% pure P-gp (Fig. 1B, lane 2). The insoluble pellet still contains half of the total P-gp, which cannot be removed at higher detergent concentrations. This extraction procedure is rapid, and as discussed below, results in the retention of a large fraction of the P-gp ATPase activity of the membrane starting material.

The Mg^{2+}-dependent ATPase activity of CH^RC5 plasma membrane displays an approximately 5-fold higher ATPase activity than membrane from the drug-sensitive parent cell line AuxB1. This additional ATPase activity is attributed to the presence of P-gp. Addition of the chemosensitizers verapamil and trifluoperazine leads to a further 2-fold stimulation in total ATPase activity. Other drug substrates, such as colchicine and vinblastine, also increase ATPase activity, but to modest levels. The observation of drug-stimulated ATPase activity suggests that there is allosteric communication between the drug binding site(s) of P-gp, which are widely assumed to be located within the hydrophobic membrane-bound peptide segments, and the ATP binding folds, which are ostensibly cytosolic domains. However, it is interesting to note that the well-known P-gp transport substrate daunomycin fails to stimulate the ATPase activity of CH^RC5 membrane significantly. The plasma membrane of Sf9 insect cells overexpressing human class I P-gp also displays high ATPase activity, which is further stimulated by drugs (Sarkadi *et al.*, 1992). In this case, stimulation by daunomycin was observed. It is possible that the host lipid environment may markedly affect the drug stimulation profiles of the P-gp ATPase (see below).

Measurements at various stages of the extraction process indicate that the S_2 fraction from CH^RC5 cells possesses a high level of ATPase activity (0.46 μmol/min/mg) compared to an identical extract from AuxB1 (0.07 μmol/min/mg). The activity of the S_2 fraction represents about 85% of the native P-gp ATPase activity (after accounting for the recovery of P-gp in S_2). Contaminating Na^+K^+-ATPase in the S_2 fraction is very low, and inhibitor studies (see below) also demonstrate the absence of significant amounts of other common membrane-bound ATPases.

BIOCHEMICAL CHARACTERIZATION OF P-GLYCOPROTEIN ATPase

Essentially all of the Mg^{2+}-ATPase activity of the S_2 fraction is attributed to P-gp, which makes this partially purified preparation extremely useful for characterization of the catalytic properties of the ATP binding domains (Doige *et al.*, 1992). Many of

Table I. Effects of Various Inhibitors on P-glycoprotein ATPase Activity[a]

Inhibitor	Type	Inhibition of P-gp ATPase	IC_{50} or maximum concentration tested
Vanadate	P-type ATPases	+	$IC_{50} = 1{\cdot}5\ \mu M$
Bafilomycin A1	V-type and other ATPases	+	$IC_{50} = 1\ \mu M$
NEM	SH reagent	+	$IC_{50} = 80–150\ \mu M$
$HgCl_2$	SH reagent	+	
p-CMBS	SH reagent	+	
EGTA	Ca^{2+}-ATPase	–	0.5 mM
Ouabain	Na^+K^+-ATPase	–	1 mM
Azide	F_oF_1-ATPase	–	2 mM
Oligomycin	F_oF_1-ATPase	–	100 ng/ml
DCCD	F_oF_1-ATPase lysosomal ATPases	–	100 μM
Molybdate	Acid phosphatases	–	10 μM
Tartrate	Acid phosphatases	–	10 mM
L-Phe	Alkaline phosphatases	–	25 mM

[a] For full details, see Doige *et al.* (1992).

the properties of the ATPase have since been confirmed using a highly purified P-gp preparation (>90% pure), prepared by lentil affinity chromatography of the S_2 fraction, which has one of the highest specific activities reported to date (1.65 μmol/min/mg; Sharom *et al.*, 1995). P-gp in CHAPS solution displays a relatively high K_M for ATP (0.4–0.9 mM), which indicates that the transporter has a low affinity for ATP compared to many other membrane-bound ATPases. Mg^{2+} ions are optimal for ATP hydrolysis; only 45% and 22% of the catalytic activity is retained when Mg^{2+} is substituted by Mn^{2+} and Co^{2+}, respectively. The V_{max} for ATP hydrolysis is increased up to 50% by the addition of verapamil and trifluoperazine. Kinetic analysis of highly purified P-gp shows that verapamil acts as a "mixed" activator, producing both an increase in V_{max} and a decrease in K_M (Sharom *et al.*, 1995). No kinetic cooperativity is evident, which suggests that the two ATP sites operate independently of each other. In general, the level of ATPase stimulation by drugs is lower than that noted for the native CH^RC5 plasma membrane, which indicates that the presence of detergent disrupts the ability of drugs to modulate catalytic activity at the ATPase site, perhaps by partially unfolding critical regions of the protein.

Investigation of the nucleotide specificity of highly purified P-gp has revealed that it is relatively selective for adenine nucleotides; other ribo- and deoxyribonucleotides are hydrolyzed at less than 10% of the rate seen for ATP and dATP. Urbatsch *et al.* (1994) have shown that ATP analogs containing a modified adenine residue, including the 8-bromo, 8-azido, and 1,N^6-etheno derivatives, are also reasonably good substrates. These results support the idea that the ATP binding sites on P-gp are of low affinity, and tolerant of adenine ring modification.

An extensive investigation of potential inhibitors of the P-gp ATPase has been carried out (Doige *et al.*, 1992; see Table I). EGTA and ouabain, inhibitors of the Ca^{2+}- and Na^+K^+-ATPases respectively, do not affect the P-gp ATPase. Inhibitors of the F_oF_1-ATPase, such as azide, oligomycin, and DCCD (also an inhibitor of lysosomal ATPases), do not alter P-gp ATPase activity in the appropriate concentration range. Activity is also unaffected by inhibitors of acid phosphatases (molybdate, tartrate) and alkaline phosphates (L-Phe). We have identified three classes of compound as inhibitors of P-gp ATPase. Orthovanadate, a classical inhibitor of many P-type ATPases with a K_i in the range 50–500 nM, abolishes P-gp ATPase activity at low μM concentrations (K_i = 1·5 μM). This result confirms that a pentacoordinate phosphate intermediate is involved in catalysis by P-gp ATPase. However, P-gp does not possess the conserved Asp residue that participates in the formation of a covalently phosphorylated reaction intermediate in the P-type ATPases. The sulfhydryl-reactive agents NEM, *p*-CMBS, and $HgCl_2$ all inhibit P-gp ATPase with K_i values in the 80–150 μM range. ATP protects the P-gp ATPase from inactivation by these compounds at two sites per molecule (Al-Shawi and Senior, 1993; Urbatsch *et al.*, 1994). The Walker A motifs of all P-gp gene classes contain a conserved Cys, and it seems likely that this residue is the target for attack by sulfhydryl reagents. The macrolide antibiotic bafilomycin A1 inhibits P-gp ATPase activity at concentrations of 0.1–10 μM, with a stoichiometry of 0.9 μmol/mg protein (Sharom *et al.*, 1995). This is at the lower end of the inhibition range characteristic of P-type ATPases (10–100 μM for the Ca^{2+}- and Na^+K^+-ATPases), but is substantially higher than the concentration needed to inhibit V-type ATPases (typically in the nM range) (Bowman *et al.*, 1988). The effect on P-gp ATPase of other members of the bafilomycin and concanamycin family of antibiotics is currently being explored. One obvious conclusion to be made from these studies is that P-gp ATPase has a unique inhibitor profile, which clearly differentiates it from other major classes of membrane-bound ATPases.

LIPID AND DETERGENT INTERACTIONS

The interactions of a membrane transporter such as P-gp with lipids and detergents are especially important if the final goal is functional isolation and reconstitution. Both the S_2 fraction (Doige *et al.*, 1993) and a highly purified P-gp preparation (Sharom *et al.*, 1995) have been used to explore these interactions. Several detergents inactivate P-gp ATPase activity at relatively low concentrations, including Triton X-100, digitonin, SDS, and deoxycholate. On the other hand, CHAPS and octylglucoside preserved the ATPase activity well at concentrations up to 5 mM. Two different approaches were used to investigate the lipid preferences of P-gp (Doige *et al.*, 1993). Incubation of partially purified P-gp in CHAPS at 23°C results in gradual loss of ATPase activity, which can be protected from inactivation by addition of asolectin and DPPE, but not various species of PC.

PS is also able to preserve ATPase activity, whereas PI is only moderately effective. When lipids are added to highly purified P-gp in CHAPs at 4°C, 3-fold concentration-dependent activation of the ATPase is observed for saturated PE species (DMPE and DPPE), while egg PC leads to 2-fold stimulation (Sharom *et al.*, 1995). Addition of PI and PS produces substantial inhibition. Thus P-gp ATPase is highly lipid-dependent, and particular species, especially saturated PE and egg PC, increase the catalytic activity. Like verapamil, DPPE acts as a mixed activator, both increasing V_{max} and decreasing K_M. It is interesting to note that PE is often found on the inner leaflet of eukaryotic plasma membranes, where it would be well positioned to interact with the ATP binding domains. When P-gp is stripped of tightly bound lipids by detergent treatment, ATPase activity is completely abolished, indicating that the ATP binding domains, although apparently cytosolic, are highly dependent on the presence of lipids for their integrity. It is possible that these domains may interact with the membrane more closely than has been envisaged. This inactivation is fully reversible by the addition of certain phospholipids. In this case, only fluid lipid mixtures with low T_m values, such as egg PC and asolectin, are able to restore catalytic activity. This finding suggests that P-gp prefers fluid lipids in its immediate environment.

FUNCTIONAL RECONSTITUTION OF P-GLYCOPROTEIN

Partially purified P-gp was reconstituted into proteoliposomes using rapid removal of detergent by gel filtration chromatography (Sharom *et al.*, 1993). The lipids chosen for reconstitution studies were egg PC and DPPE. The latter both provides the best protection against heat denaturation, and produces maximal stimulation of P-gp ATPase activity at 4°C. PE suffers from the disadvantage that it prefers to form the hexagonal or H_{II} phase in aqueous solutions. Egg PC is a natural, fluid lipid mixture, which can maintain PE in bilayer form in mixtures of the two.

The S_2 fraction is prepared from CH^RC5 plasma membrane in the presence of 20% glycerol, which was previously found to be necessary for retention of activity of several other membrane transporters. Egg PC and DPPE, solubilized in CHAPS, are mixed with the P-gp, and a short incubation is carried out at 4°C, to allow molecular mixing of the protein and lipids. After rapid chromatography on Sexphadex G-50, the turbid fractions containing lipid structures are collected, and proteoliposomes are then harvested by centrifugation. Biochemical characterization indicates a lipid : protein ratio of 45 : 1, and a trapped volume of $\sim 1\ \mu L$/mg lipid, which is typical for reconstituted preparations. QELS measurements show that the proteoliposomes consist of a bimodal population, with diameters of 0.24 and 0.8 μm. They appear to be largely unilamellar when viewed by fluorescence microscopy in the presence of a lipid-soluble probe, which is confirmed by measurement of the cryptic ATPase activity (see below). When the reconstituted proteoliposomes are examined by SDS-PAGE, P-gp is the major band present, making up at least 50% of the protein profile (Fig. 1B, lanes 3 and 4). The purity of P-gp increases substantially on reconstitution, likely as a result of preferential incorporation of P-gp into lipid bilayers, relative to the other contaminating proteins. This factor may make it unnecessary to obtain highly purified P-gp if the ultimate goal is a reconstituted model system.

CHARACTERIZATION OF DRUG TRANSPORT BY P-GLYCOPROTEIN IN RECONSTITUTED PROTEOLIPOSOMES

Colchicine was the drug of choice for transport measurements for two reasons. First, we previously characterized colchicine transport in CH^RC5 plasma membrane vesicles (Doige and Sharom, 1992), and this allows us to make direct comparisons between proteoliposomes and this system. Second, it is one of the least hydrophobic drugs in the MDR spectrum, and nonspecific partitioning of colchicine into lipid is minimal. P-gp proteoliposomes accumulate [^{3}H]colchicine in the presence of ATP, but not in its absence, reaching an equilibrium steady-state level after 2–4 min (Fig. 2). Low levels of drug uptake are seen for proteoliposomes made with an identical CHAPS extract from the parent AuxB1 cell line, or from phospholipids alone. Only P-gp molecules facing inwards, with their ATP binding folds exposed on the outer surface of the liposomes, will hydrolyze ATP and transport drug (Fig. 3). Outward-facing P-gp molecules have no access to ATP, since the vesicles are well sealed, and do not contribute to transport.

Several different approaches were used to establish that the observed drug accumulation was the result of transport into the vesicle lumen, rather

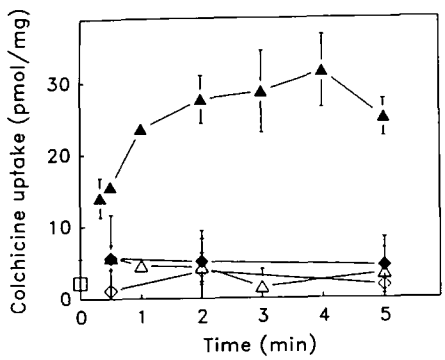

Fig. 2. Time course of colchicine uptake by reconstituted proteoliposomes containing P-gp. [^{3}H]Colchicine uptake was determined for proteoliposomes reconstituted with the S_2 fraction from either CH^RC5 (▲, △) or the parent cell line AuxB1 (◆, ◇), in the presence (closed symbols), or absence (open symbols) of ATP and a regenerating system. Liposomes of lipid alone showed background levels of drug uptake (□), which did not change with time. Colchicine concentration was 0.16 μM. For experimental details, see Sharom *et al.* (1993). Reprinted with permission.

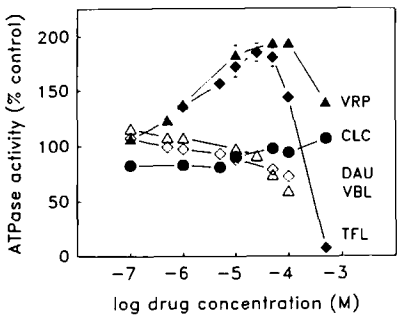

Fig. 4. Effect of drugs and chemosensitizers on P-gp ATPase activity in reconstituted proteoliposomes. ATPase activity was measured in the presence of increasing concentrations of verapamil (VRP, ▲), trifluoperazine (TFL, ◆), colchicine (CLC, ●), daunomycin (DAU, ◇), and vinblastine (VBL, △). For experimental details, see Sharom *et al.* (1993). Reprinted with permission.

than binding (Sharom *et al.*, 1993). First, ATP-dependent colchicine accumulation is osmotically sensitive; addition of hyperosmotic sucrose solutions (which leads to shrinkage, and a concomitant reduction in intravesicular volume) produces a large decrease in the amount of drug accumulated. Drug binding would not be expected to show such osmotic sensitivity. Second, permeabilization of the vesicles with low

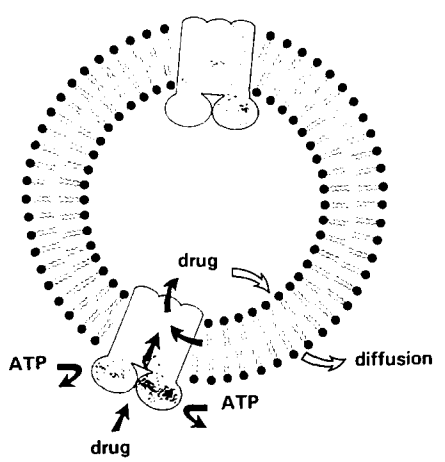

Fig. 3. Establishment of a stable equilibrium level of drug accumulation in proteoliposomes. Inward-facing P-gp molecules (i.e., with their ATP-binding folds accessible on the external surface of the vesicle) hydrolyze ATP and transport drug into the lumen, generating a concentration gradient. As drug accumulates in the interior, it diffuses out of the vesicle down the concentration gradient. Eventually a stable equilibrium is established in which drug pumping into the proteoliposome by P-gp is balanced by outward diffusion.

concentrations of CHAPS (< 3 mM) completely abolishes drug accumulation.

Colchicine transport is dependent on ATP hydrolysis, and not simply binding, since the non-hydrolyzable analog ATPγS cannot support drug uptake, even though this compound binds to the ATPase domains of P-gp with only a slightly lower affinity than ATP. In contrast to previous observations in plasma membrane vesicle systems, the presence of an ATP-regenerating system is not strictly required to observe stable drug accumulation in proteoliposomes. This observation likely reflects the fact that added ATP is not deplected by other ATPases in this model system. The initial rate of colchicine transport into the proteoliposomes is substantially higher than that noted previously for the native plasma membrane (~ 26 pmol/min/mg compared to 4.4 pmol/min/mg at 0.16 μM drug). After accounting for the different P-gp content of the two model systems, and the proportion of the transporter that faces inwards in each, it is estimated that P-gp retains ~40% of its drug transport capability after reconstitution.

Steady-state ATP-dependent colchicine uptake is saturable at around 150 μM drug, with half-maximal accumulation seen at 50 μM. Calculation of the stoichiometry of drug uptake reveals that 7.5-fold more drug is accumulated than the number of P-gp molecules present, which is again indicative of a transport rather than a binding process. Using the measured trapped volume of the proteoliposomes, we can calculate that in the presence of ATP and 50 μM external colchicine, P-gp generates a stable 5.6-fold gradient of drug. In the absence of ATP, only diffusional equilibrium of drug across the membrane takes place. Thus,

the steady-state accumulation of drug represents a balance between inward-pumping via P-gp, up a concentration gradient, and outward leakage of drug by diffusion, down a concentration gradient (Fig. 3).

Since P-gp confers resistance to a unique spectrum of drugs, the effects of other compounds on colchicine transport was examined. Vinblastine, a preferred P-gp substrate, inhibits transport at submicromolar concentrations, while daunomycin and verapamil block transport in the low micromolar range. Two drugs that are not part of the MDR spectrum, cytosine arabinoside and methotrexate, have no effect on colchicine transport. It can be concluded that P-gp in lipid bilayers retains the anticipated specificity for MDR spectrum drugs.

As well as carrying out drug transport, P-gp in proteoliposomes fully retains its ATPase activity. Those transporters that face outwards, with their ATP binding folds in the vesicle lumen, do not contribute to ATPase activity if the vesicles remain sealed (Fig. 3). This cryptic ATPase activity can be revealed by the addition of permeabilizing concentrations of CHAPs, when ATPase activity increases by 80%. This indicates that about 55% of the P-gp molecules face inwards and 45% face outwards; in other words, P-gp is reconstituted symmetrically.

The ATPase activity of reconstituted P-gp is stimulated by verapamil and trifluoperazine by approximately 2-fold (Fig. 4). Refolding of the protein, and restoration of the allosteric linkage between the substrate and ATP binding sites, apparently occurs following reintroduction of P-gp into a membrane environment. Drug stimulation of P-gp ATPase has also been reported by Ambudkar *et al.* (1992) and Shapiro and Ling (1994), for various reconstituted P-gp preparations. However, the patterns of drug stimulation induced by various drugs and chemosensitizers are different in each case. These variations may arise from modulation of the kinetic properties of P-gp by different lipid environments and/or detergents.

How does the turnover rate for drug transport by P-gp in vesicle model systems compare to that of other ABC transporters? Using initial rate measurements carried out in our laboratory with CH^RC5 membrane vesicles (DiDiodato and Sharom, unpublished data), we calculate an apparent V_{max} value (at 100 μM colchicine) of about 27 nmol/min/mg P-gp, which translates into a turnover number of around 4.8 mol/min per mol of P-gp ($k_{cat} = 0.08$ sec^{-1}). This value is comparable to that reported for another ABC transporter, histidine permease (turnover number of $\sim$ 1 mol/min in membrane vesicles and intact cells; Bishop *et al.*, 1989; Prossnitz *et al.*, 1989). A turnover number of this magnitude is likely sufficient to account for the rate of drug export observed in intact MDR cells. The high basal level of P-gp ATPase activity makes it difficult to address the issue of stoichiometry of ATP hydrolysis with respect to drug transport. Comparisons with measurements of initial rates of transport (DiDiodato, Yu and Sharom, unpublished data) show that an *additional* 4 moles of ATP are hydrolyzed for each mole of colchicine transported, in both proteoliposomes and CH^RC5 membrane vesicles. This is similar to the stoichiometry reported by Bishop *et al.* (1989) for reconstituted histidine permease.

IMPLICATIONS FOR THE EUKARYOTIC ABC TRANSPORTERS

The ATPase activity of most other integral membrane-bound ATPases, such as the Na^+K^+- and Ca^{2+}-ATPases, is tightly coupled to substrate binding and transport. In contrast, P-gp displays constitutive ATPase activity, which appears to be largely uncoupled from the transport process. It is unlikely that unidentified P-gp substrates present in the assay system are responsible for this ATPase activity, since it is observed for both a simple proteoliposome system, and purified P-gp in CHAPS, which is not a P-gp substrate (Loe and Sharom, 1993). This property of the multidrug transporter is also likely to be operative in intact MDR cells, which exhibit a high rate of ATP consumption that increases markedly after addition of verapamil (Broxterman and Pinedo, 1991). This observation raises the interesting point of whether other eukaryotic members of the ABC superfamily also share the characteristic of constitutive ATPase activity. An answer to this question will only come after more intensive study at the biochemical level of proteins such as CFTR and STE-6. The mechanism by which ATP hydrolysis is used to drive active transport by ABC proteins has not yet been addressed experimentally. It is possible that the continuous turnover of ATP plays an important role in the mechanism of transport by this group of proteins.

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